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14. ABSTRACT: In previous studies, we have shown that effective anti-tumor autoimmunity can be elicited if a tumorcell challenge is given in conjunction with homeostatic T-cell proliferation, a process occurring in response to lymphopenia and dependent on signaling by self-peptide/MHC and trophic cytokines. We are currently investigating whether this principle can be applied to mouse models of advanced breast carcinoma, and whether the anti-tumor response can be enhanced using selected T-cell subpopulations, cytokines and tumor-vaccines. The results obtained during the second year of this project indicated that (a) irradiation is more effective than T-cell depletion by antibodies in inducing anti-tumor responses mediated by homeostatic T-cell proliferation; (b) the frequency of T regulatory cells (Treg) increases during homeostatic proliferation, particularly in the presence of a growing breast carcinoma; (c) in vivo depletion of Treg cells enhances the anti-tumor effect of homeostatic T-cell proliferation on subcutaneous breast carcinoma; (d) homeostatic T-cell proliferation kinetics can be significantly accelerated by injection of IL-7 complexed with anti-IL-7 antibodies; (e) IL-7/antibody complexes potentiate the effect of homeostatic T-cell proliferation on breast carcinoma metastasis; (f) tumor cells at early apoptotic stages induce production of type I interferons by dendritic cell subsets and promote efficient antigen-cross presentation to specific T cells.

15. SUBJECT TERMS
Breast Cancer, Autoimmunity, T Cell Homeostasis, Lymphopenia

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INTRODUCTION

Breast cancer remains the second most common cause of cancer death among women. Current efforts in cancer immunotherapy focus on induction of autoimmune responses against tumor-associated antigens that are primarily encoded by normal unmutated genes. Breaking tolerance for self-antigens, however, remains a major challenge. Recent studies showed that, under lymphopenic conditions, peripheral T cells undergo "acute homeostatic proliferation" to re-establish appropriate cell numbers. Since this process depends on T cell recognition of self-peptide/MHC antigens and is accompanied by acquisition of effector functions, we hypothesized a link between lymphopenia-induced homeostatic proliferation and autoimmunity (1). We also suggested that induction of homeostatic T-cell proliferation concurrently with a tumor cell challenge may be a way to preferentially expand and activate otherwise tolerant lymphocytes and, hence, elicit effective anti-tumor autoimmunity (2). Our initial experiments with melanoma cell-challenged lymphopenic mice infused with small numbers of syngeneic polyclonal T cells indicated that this is indeed the case (3). Here, we wish to extend this observation and determine whether this approach can be used to inhibit progression of established breast tumors. Specific aims include (a) to apply the principle of homeostatic T-cell expansion to inhibit growth of established tumors in models of advanced breast cancer; (b) to enhance the efficacy of the response by manipulating the composition of the infused T cells; and (c) to potentiate the anti-tumor effect by using T cell survival and proliferation promoting cytokines, and/or by enhancing tumor-antigen presentation with efficient tumor vaccines. The results will define the role of homeostatic T-cell proliferation in tumor autoimmunity, and provide the basis for translation studies and for the design of new approaches to the treatment of breast cancer.

BODY

The results summarized below describe research accomplishments associated with tasks outlined in the approved Statement of Work.

Task 1.b. Evaluate the efficacy of homeostatic T-cell expansion on established tumors of increasing size using an ectopic model of breast cancer

In our previous report, we showed that tumor growth was inhibited when a breast carcinoma cell challenge was applied to mice undergoing homeostatic T-cell proliferation (HTP), i.e. mice previously rendered lymphopenic by sublethal irradiation (600 rad) and transfused with syngeneic T cells. In addition, since irradiation of mice with established tumors could also directly affect tumor growth, alternative means of lymphopenia-induction were explored to evaluate the effect of HTP on models of advanced breast cancer. We showed that lymphopenia and HTP could be efficiently induced by antibody-mediated T cell depletion. However, the effect of this lymphopenia-inducing approach on HTP-mediated anti-tumor responses had not been examined.

   To address this point, we compared the anti-tumor responses elicited by HTP in antibody-depleted *versus* irradiated mice. BALB/c mice (Thy1.2$^+$) were rendered (or not) lymphopenic by either anti-Thy1.2 antibodies (day -4) or irradiation (day -1), then challenged subcutaneously (s.c.) with 10^5 4T1 breast carcinoma cells (day 0), and transfused (or not) with 50×10^6 syngeneic Thy1.1$^+$ LN cells (day +1). The results confirmed our previous report that irradiation-induced lymphopenia and HTP lead to significant anti-tumor responses (**Fig 1A**). Thus, s.c. tumor growth was inhibited in irradiated mice undergoing HTP compared to controls. In addition, lung metastatic nodules and weights were reduced, and a few mice survived longer time in the group of irradiated mice in which HTP was enhanced by LN cell transfusion, compared to irradiated but non-transfused hosts (**Fig 1A**). Surprisingly, however, HTP in mice rendered lymphopenic by antibody-mediated T cell depletion was not associated with significant anti-tumor effects (**Fig 1B**). The reasons for this result are unclear, but could be due to the fact that T cell-depleting antibodies may exert their function over a longer time, leading to delayed recovery of the endogenous T cell pool. In fact, additional studies showed that the number of endogenous T cells at day 20 after tumor challenge was > 2-fold reduced in antibody-treated, compared to irradiated, mice. Thus, experiments with reduced amounts of antibodies may lead to better results. Another possibility is that, unlike antibody-treatment, irradiation depletes most immune cell subsets, some of which may play essential roles in containing proper T cell proliferation and/or activation. One of

these cell populations may be constituted by NK cells, as their depletion was necessary to improve the anti-tumor effect of adoptively transferred T cell clones combined with vaccination and IL-2 treatment (4). Therefore, the use of additional antibodies to deplete other relevant lymphocyte populations may improve the effect of T cell depletion.
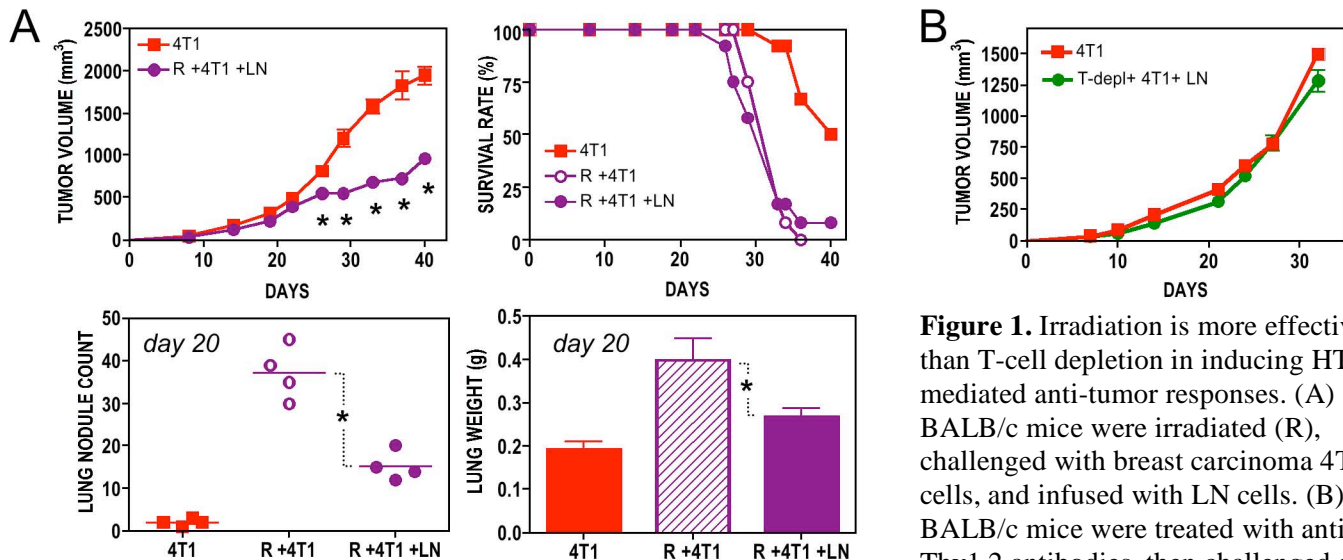

Figure 1. Irradiation is more effective than T-cell depletion in inducing HTP-mediated anti-tumor responses. (A) BALB/c mice were irradiated (R), challenged with breast carcinoma 4T1 cells, and infused with LN cells. (B) BALB/c mice were treated with anti-Thy1.2 antibodies, then challenged and infused as in A. * , p<0.05 in t-test.

Overall, the results indicated that HTP triggered by irradiation-induced lymphopenia consistently inhibits s.c. tumor growth. However, lymphopenia (which is required for HTP induction) may actually facilitate metastatic spread, at least during the early phases of lymphocyte recovery. Nonetheless, the fact that metastases were reduced in LN cell-transfused, as compared to non-transfused, lymphopenic mice, suggests that ameliorating the efficiency of T cell activation and expansion during HTP may lead to significant reduction in both s.c. tumors and metastasis. In the original proposal, we suggested that such amelioration could be achieved by various approaches, including by manipulating the composition of the expanding T cell population (e.g. depleting regulatory/suppressor T cells), by providing enhanced signaling from homeostasis related cytokines (e.g. IL-7), and by increasing the efficacy of tumor antigen presentation (e.g. using dendritic cell-based vaccines). The experiments described below were designed to address these possibilities.

Task 2.a. Determine whether depletion of regulatory/suppressor T cells from the infused cell population enhances the anti-tumor response in the ectopic model
Among T cell subsets with regulatory/suppressor activity, $CD4^+CD25^+$ T regulatory (Treg) cells are the best characterized (5, 6). These cells constitutively express the transcription factor Foxp3 and inhibit activation and expansion of antigen-engaged T cells. Accumulating evidence indicates that Treg cells not only contribute to the maintenance of immunologic self-tolerance, but also inhibit both immunologic surveillance against autologous tumors and effective tumor-specific vaccination (5-8) (9-11). In addition, Treg cells expand during HTP (12), inhibit HTP of conventional T cell subsets (13), are induced during antigen-mediated T cell activation (14) and are expanded in cancer patients and tumor bearing mice (6, 15, 16). Thus, Treg-depletion, either from the infused LN cells or directly in tumor-challenged mice, may increase the HTP-mediated anti-tumor response.

To determine how the frequency of Treg cells is modulated during HTP in the presence or absence of a growing tumor, the following experiment was conducted. Mice were irradiated (or not), challenged with 4T1 cells and transfused (or not) with syngeneic LN cells. At days 7, 15 and 23 post-challenge, 3 mice per group were sacrificed and LN and spleen cells examined by FACS for the presence of $CD4^+CD25^+Foxp3^+$ Treg cells. As shown in **Fig 2A** and **2B**, in unmanipulated BALB/c mice Treg cells consistently accounted for ~8%

of CD4 T cells. In contrast, in tumor-challenged mice Treg cells gradually increased, reaching ~22% by day 23. As the increase appeared accelerated in mice undergoing HTP, the effect of HTP was also examined in tumor-free mice. Indeed, Treg cell frequency was transiently raised during HTP, reaching ~12% by day 15, then returning to normal levels by day 20 after irradiation (**Fig 2B**). Cell enumeration also indicated that both tumor and lymphopenia-induced HTP contributed to the increase in Treg cellularity (**Fig 2C**). Moreover, in transfused mice, >50% of the Treg cells were of host origin, probably reemerging from the thymus after lymphopenia induction.
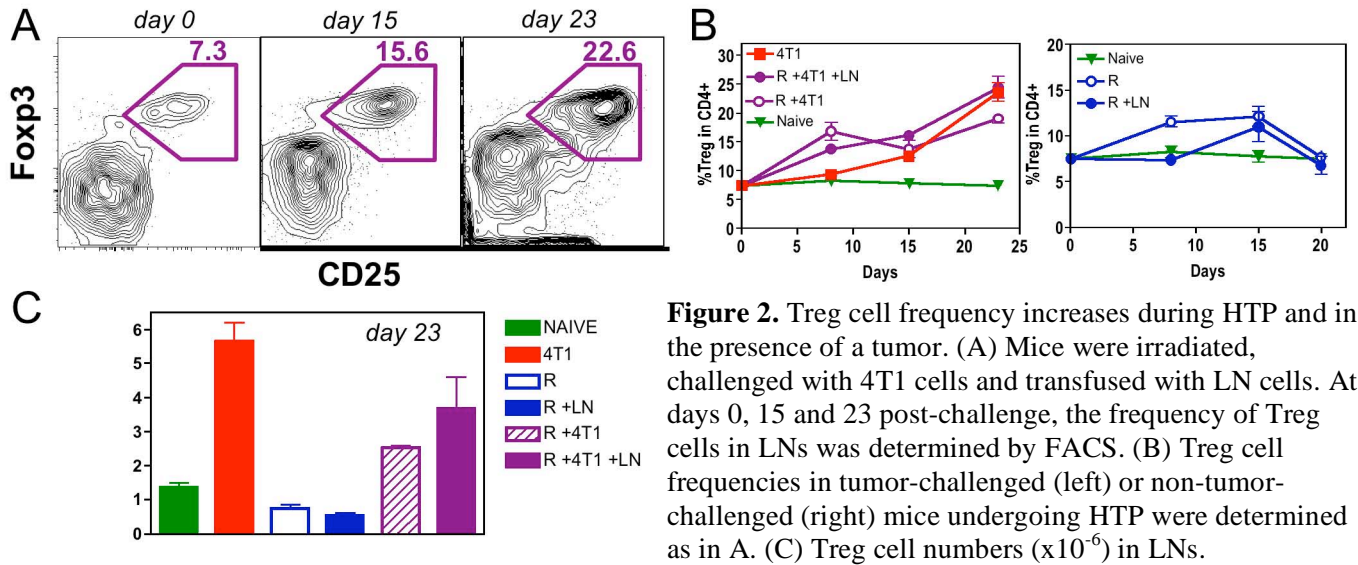

Figure 2. Treg cell frequency increases during HTP and in the presence of a tumor. (A) Mice were irradiated, challenged with 4T1 cells and transfused with LN cells. At days 0, 15 and 23 post-challenge, the frequency of Treg cells in LNs was determined by FACS. (B) Treg cell frequencies in tumor-challenged (left) or non-tumor-challenged (right) mice undergoing HTP were determined as in A. (C) Treg cell numbers ($x10^{-6}$) in LNs.

These data suggest that optimal inhibition of Treg cells would be achieved using depleting antibodies *in vivo*, rather than by depleting the donor cells before injection. Therefore, mice were irradiated (or not), challenged with 4T1 tumor cells, and injected (or not) with LN cells. In addition, mice were injected (or not) every 5 days with the anti-CD25 monoclonal antibody PC61 to deplete Treg cells as described (7, 8). The results indicated that Treg-depletion was efficient, although a residual population of Foxp3+CD25− cells remained detectable (**Fig 3A**). As previously reported, Treg-depletion significantly inhibited s.c. tumor growth in non-lymphopenic mice (**Fig 3B**). Treg-depletion also enhanced the effect of HTP on s.c. tumors, although lung metastases were only marginally affected.
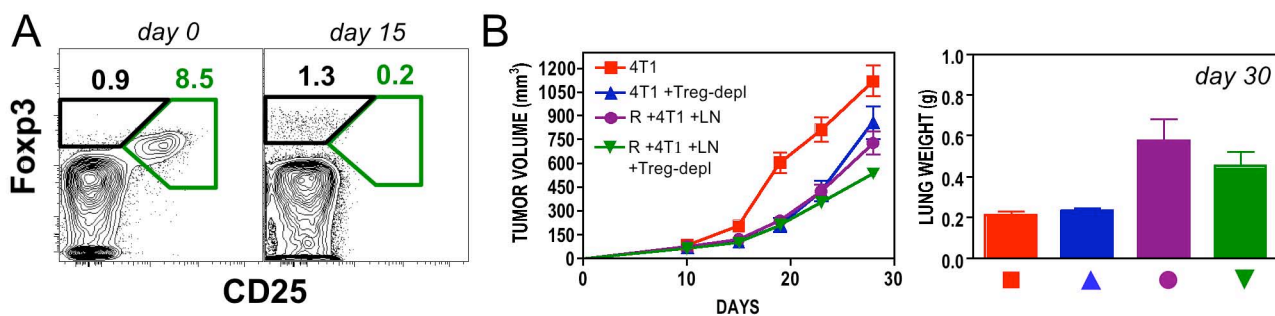
Figure 3. Treg-depletion improves the anti-tumor response mediated by HTP. Mice were irradiated (or not), challenged s.c. with 4T1 breast carcinoma cells, infused (or not) with LN cells, and treated with Treg cell-depleting anti-CD25 antibodies. (A) On days 0 and 15 the frequency of Treg cells was determined by FACS. (B) Volumes of s.c. tumors (left) at various time points and lung weight reflecting metastasis dissemination at day 30 were determined.

Task 3.b. Determine whether homeostasis-regulating cytokines enhance the anti-tumor response in the ectopic model

In our previous report we showed that the anti-tumor effect mediated by HTP is impaired in aged mice, and that this correlated with reduced HTP kinetics. We also showed, that HTP could be corrected by daily administration of the cytokine IL-7. On this basis, we suggested that, at least in aged individuals with deficient cytokine levels, anti-tumor responses could be enhanced with either IL-7 or other homeostasis-regulating cytokines. A very recent study, showed that the effect of IL-2 on HTP could be dramatically enhanced if IL-2 was complexed with certain anti-IL-2 antibodies (Ab) before injection (17). On this basis, we initiated experiments in collaboration with these investigators to examine whether this principle was also applicable to IL-7, i.e. whether IL-7/Ab complexes could be used to increase the efficiency of HTP and the associated anti-tumor responses. As we have previously observed, unlike the situation with aged individuals, administration of IL-7 alone did not enhance HTP in young mice, and the same was found for anti-IL-7 Ab (**Fig 4A**). In contrast, a significant acceleration of HTP was observed in mice injected with IL-7/Ab complexes, with the fraction of cells that proliferated within 10 days increasing from 73 to 92% for the CD4 subset and from 90 to 100% for the CD8 subset (**Fig 4A**). IL-7/Ab complexes, but not IL-7 or Ab alone, also increased HTP in non-lymphopenic hosts (**Fig 4B**), and mediated a >2-fold increase in LN T cell numbers in both lymphopenic and non-lymphopenic mice (**Fig 4C**).
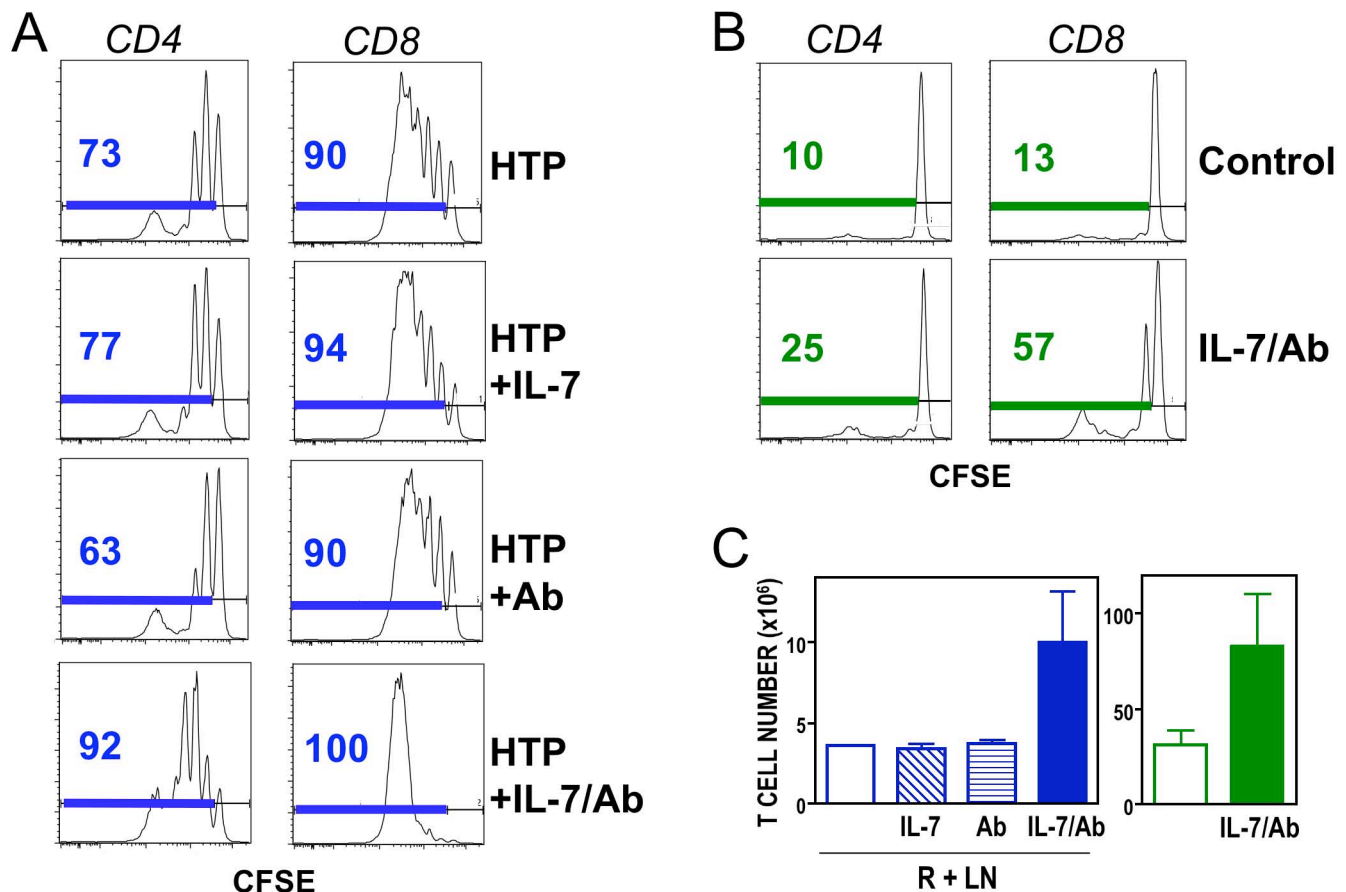

Figure 4. IL-7/Ab complexes enhance homeostatic T cell proliferation (HTP) under both lymphopenic and non-lymphopenic conditions. (A) BALB/c mice (Thy1.2) were irradiated on day -1, transfused with 50 x 10^6 CFSE-stained LN cells (Thy1.1) on day 0, and injected with IL-7, anti-IL-7 antibodies (Ab) or IL-7/Ab complexes on days 1, 4 and 7. At day 10, CFSE profiles in LNs were determined by FACS by gating on donor (Thy1.1) CD4 or CD8 T cells. (B) Non-irradiated BALB/c mice were transfused with CFSE-stained LN cells (Thy1.1), and injected with IL-7/Ab as above. CFSE profiles for donor (Thy1.1) CD4 and CD8 cells were determined on day 10. (C) Total LN T cell numbers were determined in lymphopenic (left) or non-lymphopenic (right) mice used in the experiments described in A and B, respectively.

Encouraged by these results, we examined the effect of IL-7/Ab complexes in mice challenged with the breast carcinoma 4T1 cell line. Mice were irradiated (or not), tumor challenged, transfused (or not) with 50×10^6 LN cells and injected (or not) with IL-7/Ab complexes every 3 days for 2 weeks, and then once a week for the rest of the experiment. As shown in **Fig 5**, IL-7/Ab complexes had a small, but measurable effect on s.c. tumor growth, in both lymphopenic and non-lymphopenic mice. Remarkably, however, IL-7/Ab complexes had a significant effect on lung metastasis ($p = 0.027$ in Student t-test for HTP+IL-7/Ab compared to HTP). Thus, 2 of the 6 mice had no surface metastatic nodules, and 2 other mice had < 2 nodules on day 22 post-challenge when the experiment was terminated. Current studies are confirming these results and examining the effect of these treatments at later time points.
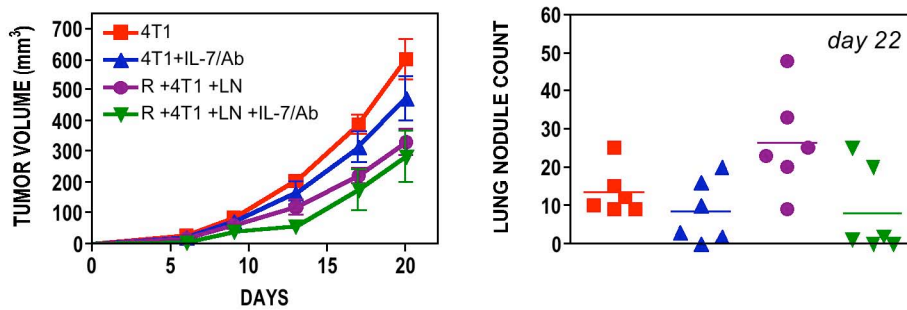

Figure 5. IL-7/Ab complexes enhance the anti-tumor response mediated by HTP. BALB/c mice were irradiated (or not), challenged with breast carcinoma 4T1 cells, transfused (or not) with 50×10^6 LN cells, and treated (or not) with IL-7/Ab complexes. Growth of s.c. tumors was followed up to day 20. On day 22, mice were sacrificed and lung metastatic nodules counted under dissection microscope.

Task 3.d. Evaluate whether dendritic cells pulsed with tumor-lysates enhance the anti-tumor response in the ectopic model

In the original application, we proposed to use tumor-lysates to stimulate dendritic cells (DC) before injection into tumor-challenged mice. However, a more efficient stimulation may be achieved using apoptotic or necrotic tumor cells in themselves. Indeed, cell apoptosis and necrosis occur in a variety of physiologic and pathologic conditions, including in response to infection and tumor growth. Thus, sensing dying cells, may be an important mechanism to alert the organism, a concept previously proposed as the "danger hypothesis" (18). Consistent with this idea, apoptotic and necrotic cells have been shown to promote strong adjuvant effects (19-21). Although the mechanisms mediating these adjuvant effects are unknown, type I interferons (IFN) produced by DCs in response to apoptotic/necrotic cell products are likely candidates. Considered the most pleiotropic among cytokines, type I IFNs encompass a large family of molecules, of which IFN-α and IFN-β (collectively referred to as IFN-α/β) are the most immunologically relevant. IFN-α/β exert multiple effects on the immune system, including activation of DCs, T cells, B cells and NK cells (22, 23). Importantly, as we have recently reviewed (24), IFN-α/β are emerging as being major effectors in autoimmunity. Thus, as anti-tumor responses are essentially autoimmune in nature, we have suggested that induction of these cytokines may be critical for effective cancer immunotherapy.

To determine whether apoptotic or necrotic cells can activate DCs, induce IFN-α/β, and promote tumor antigen cross-presentation we initially performed a series of *in vitro* and *in vivo* experiments. Splenocytes from NZW and MRL-*Fas^{lpr}* mice were treated with various doses of γ-irradiation, i.e. 1500 rad to induce early apoptosis, 3000 rad to induce advanced apoptosis/necrosis, or 0 rad as control. NZW and MRL mice are autoimmune strains available in our laboratory and were used to contain costs, although similar results were obtained in several control experiments with normal BALB/c and C57BL/6 (B6) mice. Early apoptotic, necrotic and control cells (10^6) were then used to stimulate *in vitro* syngeneic DCs (2.5×10^5) previously derived from bone marrow by stimulation with Flt3L (Flt3L-DCs) as described (25). Flow cytometry analysis

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confirmed that Flt3L-DCs contained plasmacytoid DC (pDC) subsets (CD11c$^+$ B220$^+$ PDCA-1$^+$) and conventional CD11c$^+$ B220$^-$ DC subsets (both CD24$^+$CD11b$^-$ and CD24$^-$CD11b$^+$) in the expected proportions (25, 26). In addition, as previously reported, Flt3L-DCs showed optimal responses to a variety of stimuli, including polyIC (a stimulus for TLR3), polyU complexed with the liposome DOTAP (TLR7) and CpG ODN-
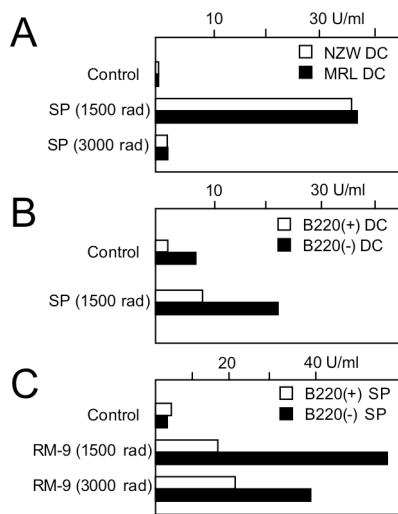

Figure 6. RM-9 carcinoma cells induce production of IFN-α/β by B220(-) DCs. (A) Flt3L-DCs from NZW and MRL-lpr autoimmune mice were stimulated *in vitro* with syngeneic irradiated spleen cells (SP). (B) Flt3L-DC from B6 mice were sorted into B220(+) and B220(-) cells and stimulated with syngeneic irradiated spleen cells. (C) Spleen cells from B6 mice were sorted into B220(+) and B220(-) cells and stimulated with irradiated RM-9 cells. IFN-α/β production was tested after 48 hrs incubation by a bioassay with a cell line expressing an ISRE-responsive element luciferase reporter construct.

2216 (TLR9) (data not shown). As shown in **Fig 6A**, splenocytes at early apoptotic stages (1500 rad), but not at advanced stages (3000 rad), induced IFN-α/β production by Flt3L-DCs. In further experiments, it was shown that sorted B220$^-$ Flt2L-DCs (containing conventional DCs), but not B220$^+$ Flt3L-DCs (containing pDCs), produced IFN-α/β in response to apoptotic cells (**Fig 6B**). To determine whether apoptotic tumor cells can also induce IFN-α/β, RM-9 carcinoma cells were used to stimulate syngeneic splenocytes. As shown in **Fig 6C**, RM-9 cells at early apoptosis stages, but less at advanced (necrotic) stages, induced IFN-α/β production. In particular, response to RM-9 cells was shown by B220$^-$ splenocytes, containing conventional DCs.

To test whether apoptotic cells can prime cytotoxic T cell immune responses *in vivo*, B6 mice were injected s.c. with 10×10^6 cells that were γ-irradiated (1500 rad) or not (control). The injected cells were derived from B6 mice expressing membrane ovalbumin (OVA) as a model antigen. After 8 days, splenocytes were obtained and restimulated *in vitro* for 6 days. The frequency of antigen-specific CD8 T cells was then examined by measuring intracellular IFN-γ production in response to the H-2K^b-binding OVA$_{257-264}$ peptide (SIINFEKL, 5 μg/ml) as described (27). As shown in **Fig 7**, strong CD8 T cell responses were induced only in mice injected with apoptotic (γ-irradiated) cells. To verify that this response is due to cross-priming by host antigen-presenting cells, the experiment was repeated using apoptotic cells from OVA-transgenic B6 mice expressing mutant H-2K^{bm1}, which is unable to present the dominant OVA$_{257-264}$ peptide (28), thereby preventing direct presentation by apoptotic cells. Similar results were obtained as in Fig 3, confirming that T cell activation occurs *via* cross-priming.

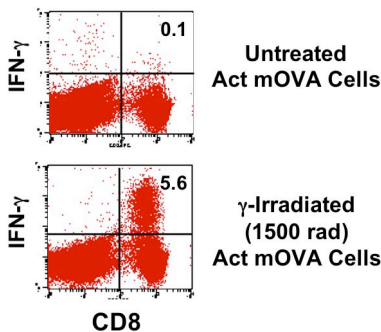**Figure 7. Apoptotic cells expressing membrane OVA induce strong effector T cell responses *in vivo*.** Spleen cells from OVA-transgenic mice were irradiated (or not) and injected s.c. in B6 mice. After 8 days, spleen cells were harvested and restimulated for 6 days. The frequency of antigen specific T cells was determined by assessing IFN-γ producing cells in response to OVA$_{257-264}$ peptide.

Overall, these results suggest that injection of early apoptotic tumor cells, or of B220-negative DCs stimulated *in vitro* with early apoptotic tumor cells, may significantly enhance tumor antigen cross-presentation and priming of specific T cells. Current studies are examining the effect of such manipulations coupled or not with HTP to prevent or treat advanced breast carcinoma and metastasis.

KEY RESEARCH ACCOMPLISHMENTS

- Irradiation is more effective than T-cell depletion by antibodies in inducing anti-tumor responses mediated by homeostatic T-cell proliferation
- The frequency of T regulatory cells (Treg) increases during homeostatic proliferation, particularly in the presence of a growing breast carcinoma
- In vivo depletion of Treg cells enhances the anti-tumor effect of homeostatic T-cell proliferation on subcutaneous breast carcinoma
- Homeostatic T-cell proliferation kinetics can be significantly accelerated by injection of IL-7 complexed with anti-IL-7 antibodies
- IL-7/antibody complexes potentiate the effect of homeostatic T-cell proliferation and inhibit breast carcinoma metastasis to the lungs
- Tumor cells at early apoptotic stages induce production of type I interferons (IFN-α/β) by dendritic cell subsets and promote efficient antigen-cross presentation to specific T cells

REPORTABLE OUTCOMES

Baccala, R., and Theofilopoulos, A.N. 2005. The new paradigm of T-cell homeostatic proliferation-induced autoimmunity. *Trends Immunol* 26:5-8.

Baccala, R., Kono, D.H., and Theofilopoulos, A.N. 2005. Interferons as pathogenic effectors in autoimmunity. *Immunol Rev* 204:9-26.

CONCLUSIONS

Because of the exquisite specificity and effectiveness of the immune system, immunotherapy is a very attractive approach for the treatment of cancer. There is, indeed, a large body of evidence indicating that, if efficiently activated, the immune system can specifically recognize and destroy syngeneic tumor cells of diverse histological origins. However, despite the advances made in recent years in identifying antigenic determinants displayed by cancer cells and devising innovative approaches, clinical success has been limited primarily by the difficulty of overcoming T-cell tolerance for tumor antigens. Based on the observation that, during lymphopenia-induced homeostatic proliferation T cells must interact with self-antigens and acquire a semi-activated state, we and others have suggested that T-cell priming through this process might overcome T-cell tolerance against tumor antigens. We have shown that the anti-tumor effect of homeostatic T-cell proliferation can be used to inhibit tumor progression in a model of breast carcinoma, is optimally promoted by irradiation-induced lymphopenia, and can be enhanced by depleting T regulatory cells and by injecting the cytokine IL-7 complexed with anti-IL-7 antibodies. Current studies are examining the mechanisms by which homeostatic proliferation, particularly under the effect of enhanced signaling by various cytokines such as IL-7 and type I interferons and optimal antigen cross-presentation, may engage tolerant T cells and promote anti-tumor responses.

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